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Downregulation of regenerating islet-derived 3 alpha (REG3A) in primary human gastric adenocarcinomas

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Abbreviations: CKAP2/TMAP, cytoskeletal associated protein 2/tumor-associated microtubule associated protein; DD-PCR, differential-display polymerase chain reaction; DLC-1, deleted in liver cancer-1; HIP/PAP, hepatocarcinoma-intestine-pancreas/pancreatitis-associated protein; REG3A, regenerating islet-derived 3 alpha

Abstract

Gastric carcinoma is considered to be one of the most prevalent cancers worldwide. We have performed differential-display polymerase chain reaction (DD-PCR) in order to compare the gene expression profile of gastric carcinoma and that of a normal stomach, in an attempt to identifiy differentially expressed genes associated with primary human gastric cancers. One of the down-regulated genes in gastric cancers was identified as regenerating islet-derived 3 alpha (REG3A), also known as hepatocarcinoma-intestine-pancreas/pancreatitis-associated protein (HIP/PAP). REG3A exhibited relatively high expression levels in normal gastric mucosa. However, REG3A was found to be down-regulated in 67% (20 out of 30 samples) of primary human gastric cancers, as determined by RT-PCR. In addition, REG3A mRNA expression was not detected in stomach cancer cell lines, SNU cells. Immunohistochemical analysis further confirmed the down-regulation of REG3A expression in primary human gastric cancers. Treatment with the demethylating agent, 5-aza-2'-deoxycytidine (5-Aza-dC) resulted in the restoration of REG3A mRNA expression in the gastric cancer cell line, indicating that the transcriptional silencing of REG3A in SNU cell lines was caused by DNA methylation. Taken together, these data indicate that REG3A is down-regulated in most primary human gastric cancer cells, and might be useful in the diagnosis of gastric cancer. Further characterization of the differentially expressed gene, REG3A, should lead to a better understanding of the changes occurring at the molecular level during the development and progression of primary human gastric cancer.

Keywords: DNA methylation; gene expression profiling; gene expression regulation, neoplastic; pancreatitis-associated protein; Reg3a protein; stomach neoplasms

Introduction

Gastric carcinoma is one of the most prevalent cancers worldwide, and constitutes a leading cause of cancer mortality in certain areas, including Korea, Japan, China, South America, and Eastern Europe (Correa, 1985, 1988). According to the classifications established by Lauren, gastric carcinoma can be divided into diffuse (poorly differentiated)- or intestinal (well differentiated)-type adenocarcinomas. Intestinal-type tumors develop mainly in glandular formations lined by large columnar cells, with well-defined cytoplasm and large nuclei. In contrast, diffuse-type tumors mostly manifest as small, rounded cells with smooth-edged nuclei and occur in small clusters or as solitary cells, exhibiting wide and diffuse spreading (Heider *et al.,* 1993; Dammrich *et al.,* 1995).

At the molecular level, all stages of carcinogenesis are, in general, associated with the differential expression of genes, a process which remains largely unclear. Many studies have implicated molecular and genetic alterations in the development and progression of gastric cancer (Tahara, 1993; Fuchs and Mayer, 1995; Tahara *et al.,* 1996).

Alterations in the expression of multiple genes involved in diverse cellular functions such as cell growth, as well as alterations in tumor suppressor genes or oncogenes, have been observed to occur in gastric cancer (Stemmermann *et al.,* 1994).

Gene amplification and over-expression of the growth factor receptors *c-erb* B2 and K-*sam* have been observed in intestinal- and diffuse-type gastric carcinoma, respectively (Ranzani *et al.,* 1990; Tahara, 1993; Werner *et al.,* 2001). The *c-met* proto-oncogene was found to be preferentially amplified in diffuse-type carcinoma (Kuniyasu *et al.,* 1992). Abnormal expression of various cell cycle regulators such as cyclins, cyclin-dependent kinases (CDK), and CDK inhibitors has been tentatively implicated in the pathogenesis of gastric cancer (Akama *et al.,* 1995). It has also been reported that the apoptotic receptors SC-1 and Fas were differentially expressed in gastric carcinoma cells of both intestinal- and diffuse-type, whereas these receptors are not detected in normal stomach mucosa. In addition, gastric tumor cells of both intestinal- and diffuse-types differed with regard to the expression of the tumor suppressor gene product p53 (Vollmers *et al.,* 1997).

Down-regulation of genes such as CA11 and KiSS-1 has also been observed in gastric carcinomas (Shiozaki *et al.,* 2001; Dhar *et al.,* 2004a). Reduced expression of E-cadherin, a cell adhesion molecule, correlated strongly with cellular dedifferentiation and glandular disintegration in primary and metastatic gastric cancers (Mayer *et al.,* 1993). In terms of the mechanism underlying this reduced gene expression, DNA methylation has been suggested to be responsible for the inactivation of various genes, such as ID4, thrombospondin-1, cyclooxygenase-2, and XIAP-associated factor 1 in gastric cancers (Byun *et al.,* 2003; Chan *et al.,* 2003; Hur *et al.,* 2003; Oue *et al.,* 2003).

The knowledge on the pathologically altered gene expression is crucial to understanding the tumorigenesis of primary human gastric cancers. In order to determine the molecular mechanisms underlying the development of gastric cancer, we performed differential-display polymerase chain reaction (DD-PCR) to compare patterns of gene expression between primary gastric cancer tissues and the corresponding normal gastric mucosa. DD-PCR analysis is able to detect quantitative changes in the gene expression of different cell populations simultaneously, and is not restricted only to the analysis of known genes. Multiple genes were identified by DD-PCR to be differentially expressed in primary gastric cancer tissues. Previously, we had also reported the cloning and characterization of a noble up-regulated gene, CKAP2/TMAP (Bae *et al.,* 2003).

Here, we report regenerating islet-derived 3 alpha (REG3A) to be a gene which is dramatically down-regulated in primary human gastric cancers. REG3A was originally identified as a pancreatitis-associated protein (PAP) released by the acini during acute pancreatitis, and is a secreted C-type lectin protein, which has been reported to be upregulated in primary hepatocellular carcinomas (Keim *et al.,* 1991; Lasserre *et al.,* 1992*,* 1994). Several functional studies demonstrated that REG3A may be involved in cell recognition and adhesion, and also in the protection of cells from oxidative stress-induced apoptosis (Christa *et al.,* 1996; Ortiz *et al.,* 1998).

Our results showed that REG3A mRNA expression is significantly down-regulated in gastric cancer mucosa, and REG3A protein was expressed only in normal mucosa, and not in gastric tumor tissues, suggesting that the downregulation of REG3A occurs frequently in human gastric cancers and might be useful for a gastric cancer marker. Treatment with the agent 5-aza-2'-deoxycytidine (5-Aza-dC) resulted in the restoration of REG3A transcription in the REG3A negative SNU cell lines, indicating that the silencing of the REG3A gene in these cell lines was caused by DNA methylation.

Materials and Methods

Clinical samples

A total of 30 tumor and adjacent normal tissue samples were collected at the time of dissection from patients with gastric cancer at the Seoul National University Hospital and Samsung Medical Center (SMC). The biopsies were frozen and stored at $-80^{o}C$ until preparation of total RNA, as described below. 24 male and 6 female patients with gastric cancer were included in the study, ranging in age from 33 to 74 years. All tumor tissues were histologically confirmed to be from gastric tumors. Clinical data are shown in Table 1.

Cell culture and materials

Cells of the stomach cancer cell line SNU were purchased from the Korean Cell Line Bank (Seoul, Korea), and maintained in RPMI 1640 with 10% (v/v) FBS (Invitrogen, Carlsbad, CA). Hepatocarcinoma cell line Hep3B cells were cultured in DMEM supplemented with 10% (v/v) FBS (Invitrogen). Cell lines were maintained in a humidified chamber at 5% CO_2, at $37^{o}C$. Anti-REG3A polyclonal antibody was a gift from Laurence

Table 1. Histopathology of gastric adenocarcinomas and REG3A expression*.

Patient	Normal	Cancer	Gender	Age	Location	Grade	Lauren	LN no
x11	++	−	M	56	Antrum	MD	Intestinal	9
x24	+++	−	M	58	Antrum	MD	Intestinal	8
x26	+++	−	M	38	Antrum	PD	Diffuse	5
x27	+++	−	F	64	Antrum	PD	Intestinal	0
x40	+	−	M	69	Body	muc	Intestinal	10
y23	−	−	M	36	Body	UD	ND	1
y34	+++	−	M	53	Antrum	MD	Intestinal	3
x2	+++	−	M	66	Body	muc	Diffuse	29
x1	+	−	M	48	Antrum	MD	Intestinal	12
y16	+	−	M	62	Antrum and body	muc	ND	7
y24	+++	−	M	46	Antrum	PD	Intestinal	2
y27	+++	−	M	67	Body	MD	ND	11
y30	++	−	M	63	Antrum and body	muc	Intestinal	28
y37	+	−	F	59	Body	MD	ND	0
y38	++	−	F	65	Antrum and body	PD	Diffuse	0
y4	++	−	M	62	ND	PD	Diffuse	8
x10	−	+	M	49	Antrum	PD	Intestinal	6
x22	++	+	M	40	Body	MD	ND	2
y14	+	−+	F	74	Body	muc	Intestinal	0
y36	+++	+	M	59	Body	WD	ND	1
x25	+++	+	M	61	Antrum and body	SRC	Intestinal	0
x37	+	+	M	81	Antrum	MD	Intestinal	13
y17	+	+	M	73	Body	MD	Diffuse	0
y25	+	+	M	76	Antrum	PD	Diffuse	7
x15	+++	+	M	33	Antrum and body	PD	Diffuse	0
y2	+	+	F	57	Body	PD	Intestinal	0
x20	+	++	M	57	Body	MD	Diffuse	11
y12	+	++	F	72	Antrum	PD	Intestinal	4
y8	+	++	M	58	Antrum	MD	Intestinal	1
x3	++	++	M	66	Body	MD	Intestinal	2

*(−), negative; (+), weak; (++), moderate, (+++), intensive expression of REG3A; M, male; F, female; MD, moderately differentiated; PD, poorly differentiated; muc, mucosal; WD, well-differentiated; SRC, signet-ring cell; Lauren, Lauren classification; LN no, positive lymph node number.

Christa (Institutes Necker-Pasteur, Universite Paris, France) (Christa *et al.,* 1996). SNU cells were seeded, allowed to attach over a 24-h period, and treated for 4 days with the demethylating reagent 5-Aza-dC (Sigma, St. Louis, MO) at final concentrations of 0.5, 1, or 5 μM. DMSO (Sigma) was used as a control for nonspecific solvent effects on cells. At the end of the treatment period, the medium was removed, and the RNA was extracted.

Differential-display polymerase chain reaction

DD-PCR was performed as previously described (Bae *et al.,* 2003). In brief, total cellular RNA was extracted from the mucosa of stomach cancer tissues and adjacent normal tissue using the ToTALLY RNA total RNA isolation kit (Ambion, TX), according to the manufacturer's directions. Total RNA was digested with RNase-free DNase (Invitrogen) in the presence of RNasin (Promega, Madison, WI) in order to remove residual genomic DNA before cDNA synthesis. The absence of genomic DNA contamination was verified by PCR using genomic GAPDH specific primers. DD-PCR was performed with the RNAimage kit (GeneHunter, Nashville, TN) in accordance with the manufacturer's instructions.

cDNA bands representing differentially expressed mRNAs were excised from the gels, and PCR was performed with the original set of DD-PCR primers. Purified PCR products were cloned into pCR 2.1-TOPO vectors (Invitrogen), according to the manufacturer's instructions. Clones were sequenced with the BigDye Terminator cycle sequencing ready reaction DNA sequencing Kit (Perkin-Elmer, Hayward, CA) and an

automated DNA sequencer (Applied Biosystems, Foster City, CA). cDNA sequences were analyzed with the National Center for Biotechnology Information BLAST server.

RT-PCR

Semi-quantitative RT-PCR was performed on gastric tumor and normal stomach tissues from 30 gastric cancer patients, and SNU and hepatoma cells. Total RNA was extracted from stomach tissues and SNU cells with either the ToTALLY RNA total RNA isolation kit (Ambion) or Trizol (Invitrogen). cDNA was synthesized with M-MLV thermostable reverse transcriptase (Invitrogen) using oligodT. The cDNA was amplified with primers for REG3A, DLC-1 (deleted in liver cancer), or GAPDH as an internal control. A 268-bp fragment from REG3A mRNA was amplified using an upstream primer, 5'-gtatcttggatgctgctttc-3' and a downstream primer, 5'-agctgttaccaatgctcttc-3'. Thermal cycling parameters were 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min for 30 cycles, followed by an additional 7 min at 72°C. PCR was performed with GAPDH as a control, with the upstream primer, 5'-cttcaacagcgacacccactcctc-3', and the downstream primer, 5'-ggcccctcccctcttcaa-3' for 25 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min. DLC-1 was also amplified, with the upstream primer, 5'-ggacaccatgatcctaacac-3' and the downstream primer, 5'-ctcatcctcgtctgaatcgt-3' (Kim *et al.,* 2003).

Real-time quantitative RT-PCR

REG3A and GAPDH mRNA levels were determined by quantitative real-time RT-PCR, and primers were utilized as described in RT-PCR analysis. Reactions were performed in 50 μl volumes containing, SYBR Green PCR master mix (Perkin-Elmer Biosystems). Real-time PCR was performed using a GeneAmp PCR System 9600 (Perkin-Elmer Biosystems) in 96-well optical plates. Thermal cycling conditions were as follows: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 2 min. Data were collected using the ABI analytical thermal cycler. RNA expression was calculated based on a relative standard curve, representing 10-fold dilutions of REG3A PCR products. The parameter threshold cycle was defined as the fractional cycle number at which the fluorescence generated by cleavage of the probe passes a fixed threshold above the baseline value. The target gene copy number in the unknown samples was quantified by measuring threshold cycle, and by using a standard curve.

Immunohistochemistry

Serial sections of formalin-fixed, paraffin-embedded biopsy samples were obtained for immunohistochemical analysis from the Department of Diagnostic Pathology at Samsung Medical Center. Appropriate institutional approval was obtained for all human tissue studies. Sections were immersed in xylene to effect the removal of paraffin, and were then rehydrated with graded alcohol. Epitope retrieval was performed in 10 mM citrate buffer (pH 6.0) for 20 min at 15 psi and 120°C. Endogenous peroxidase activity was blocked by the incubation of the sections in 3% (v/v) hydrogen peroxide. After blocking with normal horse serum (Vector Laboratories, Burlingame, CA), the sections were incubated with anti-HIP Ab overnight at 4°C, followed by incubation with biotinylated horse anti-rabbit IgG, and then avidin-biotin complex. The sections were developed in stable diaminobenzidine (Research Genetics, Huntsville, AL), and then counterstained with hematoxylin before mounting for microscopy.

Results

REG3A mRNA expression is dysregulated in primary human gastric cancers

The gene expression profile of gastric cancer was compared with that of normal stomach tissue by DD-PCR analysis. In order to obviate the effects of individual variations in gene expression, pairs of primary gastric adenocarcinoma and matched normal mucosa from the same individual were surgically resected from patients' stomachs. The mucosal area was carefully removed, in order to minimize stromal contamination. Signal intensities of all genes were evaluated and normalized to those of housekeeping genes. Signals with more than a 2-fold difference between cancer and normal tissues were considered to have been differentially expressed, and those cDNAs clearly up- or down-regulated in at least 3 out of 5 different patients were subjected to further cloning and characterization.

We selected a cDNA band of approximately 140 bp's in size, which was either undetectable or expressed at relatively low levels in all of the 5 gastric cancer tissues, while being expressed normally in the matched adjacent normal gastric mucosa (Figure 1). Sequence analysis revealed that the cDNAs were part of REG3A, a family in the calcium dependent (C-type) lectin superfamily,

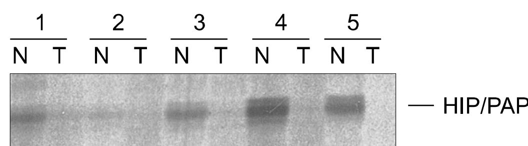

Figure 1. mRNA differential display of five gastric cancer tissues (T) and matching adjacent gastric mucosa (N). DD-PCR was performed using total RNA from gastric cancer and normal mucosa, and PCR products were resolved on 5% polyacrylamide/8M urea gel. Arrows indicate the positions of the differentially expressed cDNA fragment, REG3A. Note that REG3A mRNA expression was down-regulated in the gastric cancer mucosa compared to normal mucosa.

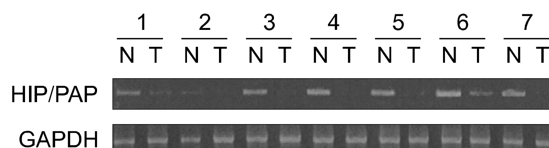

Figure 2. REG3A mRNA expression is down-regulated in primary human gastric cancers. RT-PCR was performed on 30 gastric cancer and normal stomach pairs. GAPDH was used as a control for cDNA synthesis. T and N indicate samples of histological tumor and adjacent normal tissues, respectively. Note that REG3A mRNA expression was down-regulated in the gastric cancer mucosa compared to normal mucosa.

which has been reported to be expressed in the small intestine and pancreas (Lasserre *et al.,* 1992; Christa *et al.,* 1994, 1996).

REG3A mRNA expression is down-regulated in primary human gastric cancers

REG3A cDNA was then selected for further verification of differential expression by semi-quantitative RT-PCR and quantitative real-time RT-PCR. Using specific primers designed for the REG3A cDNA clone, semi-quantitative RT-PCR was performed on 30 gastric cancer and normal stomach pairs. A representative result of this RT-PCR analysis is shown in Figure 2, which shows that REG3A mRNA was undetected in 6 out of 7 cancer tissues, and expression was clearly reduced in 1 tumor. The expression level of REG3A, as estimated by RT-PCR, is summarized in Table 1. In 20 out of 30 samples analyzed, REG3A expression levels were markedly reduced, while GAPDH signals remained similar across all lanes, confirming the result obtained from the DD-PCR study (Figure 2 and Table 1).

Quantitative real-time RT-PCR confirmed the down-regulation of REG3A in the gastric cancer mucosa. No REG3A mRNA expression was detected in 4 gastric cancer tissues, while a modest level of REG3A expression was observed in the corresponding normal tissues. In another 4 cancer tissues, the level of REG3A mRNA decreased to less than 20% of that observed in normal mucosa, while no apparent differences were observed in 2 patients (data not shown).

Detailed patient histories, and the level of REG3A expression estimated by RT-PCR, are summarized in Table 1. No significant relationship was found to exist between REG3A down-regulation and the differentiation status (or Lauren classification) of carcinoma. Collectively, our results indicate that the loss or abnormal reduction of REG3A is a frequent event in human gastric cancers.

We then investigated REG3A mRNA expression

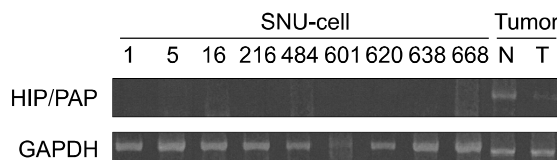

Figure 3. Expression of REG3A is absent in SNU human stomach cancer cell lines. RT-PCR was performed on 9 SNU stomach cancer cell lines and a hepatoma cell line, Hep3B. GAPDH was used as a control for cDNA synthesis. T and N indicate samples of histological tumor and adjacent normal tissues in gastric cancer patient, respectively. SNU; stomach cancer cell line. Note that REG3A mRNA expression was not observed in the human stomach cancer cell lines tested.

in human gastric cancer cell lines. 9 stomach cancer cell lines were assessed with regard to REG3A expression by semi-quantitative RT-PCR, in which GAPDH was employed as a control for both cDNA quality and efficiency of the PCR amplification. As shown in Figure 3, the REG3A transcript was undetectable in all gastric cancer cell lines (Figure 3), indicating that REG3A mRNA expression was either completely down-regulated or silenced in the stomach cancer cell lines.

REG3A protein is differentially expressed in primary human gastric tumors

Since REG3A mRNA expression was reported to be marginal in human stomach tissues (Shyamsundar *et al.,* 2005), we investigated whether REG3A protein could be detected in gastric tumor tissues. The representative immunohistochemical staining image was shown in Figure 4. REG3A was predominantly stained in non-neoplastic epithelial cells in the pyloric glands and foveolar portion of the cytoplasm, indicating that REG3A is expressed in normal stomach mucosa (Figure 4, left half). In contrast, stomach carcinoma cells demonstrated little or no staining for REG3A (Figure 4, right half). Immunohistochemical analysis further corroborated that REG3A was down-regulated in gastric cancer tissues, whereas it was expressed in the

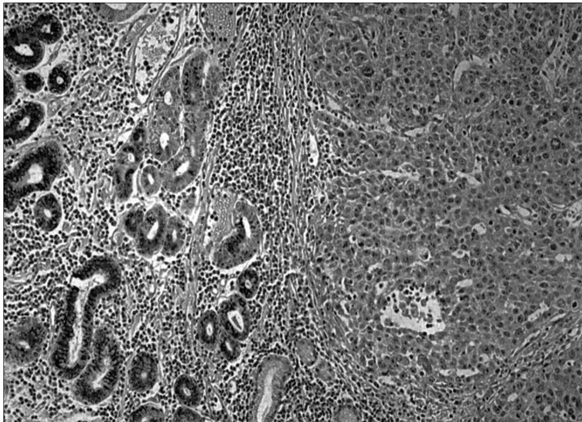

Figure 4. REG3A protein expression in gastric mucosa. Paraffin section of a gastric tumor tissue was stained with anti-REG3A polyclonal sera. The non-neoplastic epithelial cells in the pyloric glands and foveolar portion (left half) showed intense staining in the cytoplasm, while the carcinoma (right half) is completely negative for REG3A staining. (magnification, × 200).

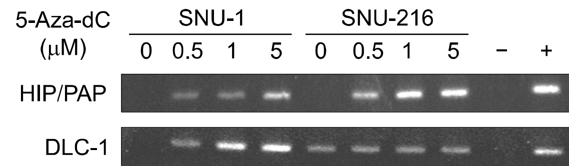

Figure 5. REG3A gene expression was restored after treatment with 5-Aza-dC. SNU-1 and -216 cells were treated for 4 days with DMSO (vehicle), 0.5, 1, or 5 μM of 5-Aza-dC. The re-expression of REG3A was detected by RT-PCR analysis. DLC-1 was also amplified, the RT-PCR primers and conditions of which are described by Kim *et al.,* 2003. −, H_2O as a negative control; +, Human stomach cDNA as a positive control.

matched adjacent normal gastric mucosa. Thus, the immunohistochemical data were consistent with the mRNA expression data obtained by differential display and RT-PCR. Based on RT-PCR and immunohistochemical staining, REG3A expression was markedly reduced or undetectable in 20 out of 30 primary cancer tissues, demonstrating that downregulation of REG3A expression is common in primary gastric cancer tissues.

REG3A expression was restored in REG3A negative cell lines by 5-Aza-dC

The demethylating agent 5-Aza-dC, a methyltransferase inhibitor, was used to investigate whether REG3A expression could be restored in REG3A negative cell lines. Two REG3A negative cell lines, SNU-1 and 216, were treated with 5-Aza-dC for 4 days at various concentrations, and REG3A mRNA expression levels were analyzed. As shown in Figure 5, REG3A mRNA expression was induced at a concentration of 0.5 μM 5-Aza-dC, and the level of REG3A mRNA expression increased in a dose-dependent manner. DLC-1 gene expression was analyzed by RT-PCR both before and after treatment of SNU cells with 5-Aza-dC (Figure 5) as a positive control. 5-Aza-dC treatment restored REG3A expression in the SNU cells, indicating that REG3A is transcriptionally silenced by DNA methylation.

Discussion

As altered gene expression is a common feature of neoplastic cells, multiple genes have been reported to be differentially expressed in tumor tissues. It can be postulated that such genes might be involved in the cellular transformation and/or phenotype of transformed cells. In order to gain understanding into the molecular events underlying the development of gastric cancer, we isolated the genes which are dysregulated in primary human gastric cancer tissues, using DD-PCR. In the present study, one of the down-regulated genes in primary human gastric cancers, namely REG3A, was selected for further analysis. We were interested in the REG3A gene because it had been previously reported to be up-regulated in liver cancers. RT-PCR (Figure 2) and real-time PCR (data not shown) analysis clearly demonstrated that REG3A expression was frequently either absent or dramatically reduced in the majority of the primary gastric cancer tissues, i.e., 20 out of 30, compared with the corresponding normal stomach mucosa. Immunohistochemistry (Figure 4) revealed that REG3A protein was expressed in normal gastric mucosa and well correlated with mRNA levels measured by RT-PCR (Figure 4). Non-neoplastic epithelial cells in the pyloric glands and foveolar portion exhibited intense staining in the cytoplasm, while carcinoma cells were completely negative for stain. In addition, REG3A expression was completely silenced in both stomach cancer cell lines (Figure 3), suggesting that the inactivation of REG3A occurs quite frequently during the development of human gastric cancer.

REG3A is a member of Reg protein family and other members of Reg protein family are also related with human gastric cancers. REG1 overexpression has been reported to be related with poor prognosis of gastric cancers (Yonemura *et al.,* 2003; Dhar *et al.,* 2004b). Overexpression of REGIV, another member of Reg protein family has been reported in certain types of human gastric cancers (Oue *et al.,* 2005). REG1 expression in gastric mucosa was mainly in enterochromaffin like

cells (Kinoshita *et al.,* 2004) and REGIV overexpression was detected only in gastric cancers having neuroendocrine differentiation (Oue *et al.,* 2005). In contrast to REG1 and REGIV, REG3A is frequently downregulated in human gastric cancers according to our data and this discrepancy might suggest the distinct role of REG3A in gastric mucosa.

The loss or down-regulation of REG3A mRNA expression in stomach cancer cell lines was associated with DNA methylation (Figure 5). An epigenetic mechanism involving DNA methylation has been shown to be responsible for the silencing of tumor associated genes in a variety of human cancers (Park *et al.,* 2007). Indeed, several studies have shown that the methylation of the CpG island results in the inactivation of various genes, for example, cell adherence (E-cadherin), and cell cycle regulation (pRB, $p15^{INK4b}$, $p16^{INK4a}$) (Herman *et al.,* 1996; Strizaker *et al.,* 1997; Song *et al.,* 2000). DNA methylation has also been associated with the inactivation of ID4, thrombospondin-1, cyclooxygenase-2, and XIAP-associated factor 1 in gastric cancers (Byun *et al.,* 2003; Chan *et al.,* 2003; Hur *et al.,* 2003; Oue *et al.,* 2003). Genes silenced by DNA methylation can be restored by treatment with 5-Aza-dC, a well-established DNA methyltransferase inhibitor (Jones and Tayler, 1980). REG3A gene expression was also restored by 5-Aza-dC in human stomach cancer cell lines, indicating that REG3A is transcriptionally silenced by DNA methylation. Further study is required to determine whether REG3A undergoes epigenetic silencing in gastric cancer cell lines and primary carcinoma via aberrant CpG site hypermethylation of the gene promoter. The mechanism underlying the down-regulation of REG3A in human gastric cancer will later be investigated in detail.

REG3A is a family in the calcium dependent (C-type) lectin superfamily consisting of a carbohydrate recognition domain (CRD) of 138 amino acids which is linked to a signal peptide (Lasserre *et al.,* 1992). The CRD of REG3A protein has been shown to have a specific lactose binding activity (Christa *et al.,* 1994). The C-type lectin family includes selectin cell adhesion molecules, endocytic receptors, and secreted molecules found in the extracellular matrix, and participates in cell surface recognition mediated by protein-carbohydrate interactions (Lasserre *et al.,* 1992, 1994). REG3A has been reported to bind to laminin-1 and fibronectin, and has also been implicated in cell to cell interaction, differentiation, and metastasis (Christa *et al.,* 1994, 1996). Functional studies have indicated that REG3A may play a role in the adhesion of cells to the extracellular matrix, and also in the protection of cells from oxidative stress-induced apoptosis (Christa *et al.,* 1996; Ortiz *et al.,* 1998). Recently, it has been reported that REG3A stimulates liver regeneration after partial hepatectomy in transgenic mice (Simon *et al.,* 2003). REG3A increased hepatocyte DNA synthesis and protected these cells against TNF-α plus actinomycin-D-induced apoptosis, implying that REG3A possesses mitogenic and anti-apoptotic abilities in hepatocytes, and consequently functions as a growth factor (Simon *et al.,* 2003).

REG3A was previously reported to be up-regulated in 25% of primary liver cancers, although REG3A expression was not detected in normal liver tissue (Lasserre *et al.,* 1992). REG3A expression has been reported not only in liver cancers, but also in normal tissues such as the pancreas, small intestine, duodenal, jejunal, and ileal mucosa, displaying a number of positive cells in the bottom of the crypt. This pattern is consistent with that of paneth cells, suggesting that REG3A plays some role in these normal tissues (Christa *et al.,* 1994, 1996). REG3A gene expression was, however, undetectable in other adult normal tissues, such as the colon, brain, kidney, and lung. The present study demonstrated that REG3A was also expressed in the human stomach, but down-regulated to a significant extent in the majority of primary human gastric carcinoma (67%; 20 out of 30). This finding was in contrast to a previous study reporting the modest up-regulation of REG3A in primary human hepatoma (24%; 7 out of 29). This difference in REG3A expression between gastric carcinoma and hepatoma may be due to tissue-specific dependent expression. Although little is currently known regarding the underlying mechanisms, tissue specificity is one of the most important aspects of the genetic modification of tumor susceptibility (Coates *et al.,* 2003). In human gastric cancer cell lines, REG3A promoter seems to be hypermethylated, resulting in the transcriptional silencing of REG3A, although the CpG island hypermethylation of REG3A has not yet been demonstrated by bisulfite modified sequencing analysis. We presume that the epigenetic inactivation of REG3A occurs during tumorigenesis in gastric cancer, rather than the transcriptional silencing of REG3A promoting gastric cancer development and progression, as REG3A expression was observed in the absence of epigenetic inactivation in human hepatoma carcinoma. Further detailed studies regarding the variability of REG3A gene regulation in tumors of different type of tissue are clearly warranted.

The investigation of molecular and genetic alterations in gastric cancers has provided useful

insights into the pathogenesis of the disease and provided invaluable tools for the differential diagnosis of primary tumors. For example, gene amplification and altered expression of *c-erb* B2 is selectively found in intestinal-type gastric cancer, and may serve as a prognostic marker for tumor invasion and lymph node metastasis (Becker *et al.,* 2000). Genetic mutations of E-cadherin have been reported to be responsible for a dominantly inherited form of gastric cancer, and this might improve conventional diagnosis (Becker *et al.,* 1994; Oda *et al.,* 1994). Cyclin E overexpression can be strongly correlated with staging, invasiveness, and proliferation, and may serve as a marker for aggressiveness (Akama *et al.,* 1995). Although additional studies are required for the characterization of the biological significance of REG3A inactivation in gastric tumorigenesis, our study suggests that the down-regulation of REG3A might be used as a molecular marker for the detection of human gastric cancers. The utility of REG3A as a potential diagnostic marker for human primary gastric tumor should be evaluated in additional studies.

In the present study, we investigated the regulation of REG3A expression, and the mechanisms underlying its regulation in human gastric cancers. REG3A is down-regulated in the majority of primary human gastric cancers and REG3A expression was silenced in a subset of gastric cancer cell lines, indicating that the loss of expression may be associated with DNA methylation. Characterization of the role of REG3A in the development of tumors should lead to a better understanding of the changes occurring at the molecular level during the development and progression of primary human gastric cancer.

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